# Morphological and structural observation of the nuclei during spermiogenesis in *Gampsocleis gratiosa* (Orthoptera, Tettigoniidae)

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**Abstract**: 【Aim】 The distinctive arrow-shaped morphology and large size of the spermatozoa of *Gampsocleis gratiosa* makes it a useful model to explore spermiogenesis in Tettigoniidae. In order to better understand the mechanism of spermiogenesis, especially the mechanism of nucleus morphogenesis and arrow-shaped acrosome formation in katydids, the nuclei of spermatid and spermatozoa of *G. gratiosa* were observed in the present study. 【Methods】 The testes of *G. gratiosa* in sexually mature males were used as the materials. We observed the nuclei of spermatid and spermatozoa by transmission electron microscopy, ordinary optical microscopy, fluorescence microscopy and with H&E and DAPI (DNA specific probe) staining method. 【Results】 According to the morphology and structure, the nuclei during spermiogenesis of *G. gratiosa* were divided into four phases: rounded, leaf, prismatic and mature phases. During the spermiogenesis of *G. gratiosa*, there are two globular structures at the rounded phase, one is the nucleus and the other is pro-acrosome. The cytoplasm droplet, which would be derelict along the tail of spermatozoa at the mature phase, contains DNA. 【Conclusion】 The observation revealed that spermatid nucleus of *G. gratiosa* undergoes dramatic morphological changes during its spermiogenesis. The nucleus shaping is driven by cytoskeletal microtubules, and the chromatin reorganization is together with nucleus shaping. This study will be the foundation to clarify the molecular mechanism of spermiogenesis in Orthoptera.

**Key words**: Orthoptera; *Gampsocleis gratiosa*; nucleus; pro-acrosome; ultrastructure; H&E staining; DAPI; spermiogenesis

## 1 INTRODUCTION

Spermiogenesis is the last phase of spermatogenesis, in which spherical spermatids turn into elongated spermatozoa in insects. It is a dynamic process that includes existing organelle reorganization and new structure formation (Chapman, 1998; Klowden, 2007). During the process of spermiogenesis, the dynamics of nucleus is particularly remarkable (Sun and Yang, 2010; Brill and Wolfner, 2012; Fabian and Brill, 2012).

In insects, the most thorough research on the dynamics of nucleus during spermiogenesis is in *Drosophila melanogaster* (Fabian and Brill, 2012). The spermiogenesis of *D. melanogaster* was described at ultrastructural level many years ago (Shoup, 1967). Then Fabian and Brill concluded that as the nuclei elongate, they go through leaf, early canoe, late canoe and needle-shaped stages. The dense bodies and the acrosomes elongate together with the nuclei (Fabian and Brill, 2012). The spermiogenesis of other insects was also studied by many researchers, but they simply described the ultrastructure of spermatozoa nucleus, not giving completive summary for its dynamic changes in the process of spermiogenesis (Gall and Bjork, 1958; Kaye and McMaster-Kaye, 1966; Baccetti, 1987; Xi, 1995; Xi and Zheng, 1995; Du *et al.*, 2003).

In Orthoptera, the sperms have diverse morphological characteristics (Kaye, 1962; Baccetti, 1987; Xi, 1995; Birkhead *et al.*, 2009), and in superfamily Tettigonioidea the sperms are larger and more complex. Some Tettigoniidae species show large acrosomes with an arrow-shaped morphology (Guerra and Esponda, 1999), for example, *G. gratiosa* (Wang *et al.*, 2010).

*G. gratiosa* is widely distributed in the north China, which is collected and raised by fans. The distinctive morphology and large size of the sperm of

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*G. gratiosa* make it a useful model to explore spermiogenesis in Tettigoniidae (Zhou *et al.*, 2012). In this study we mainly focus on the morphology and ultrastructure of nucleus during spermiogenesis. Furthermore, we attempt to define the dynamic process of nucleus during spermiogenesis through this study.

# 2 MATERIALS AND METHODS

## 2.1 Test insects

Live adult males of *G. gratiosa*, which inhabited in grass and bush, being used in this study were collected from Shunping in Hebei province of China in August 2013. After being gathered to the laboratory, *G. gratiosa* specimens were grown under controlled temperature (30 ± 1℃) and photoperiod (12L: 12D). All investigations were carried out on sexually mature males.

## 2.2 Light microscopy

Testes were fixed in Bouin's fluid for 24 h at room temperature. Specimens were then thoroughly dehydrated, and embedded in paraffin. Serial sections of 7 μm were mounted on glass slides. After deparaffination with xylene and rehydration, some sections were stained with hematoxylin and eosin (H&E) staining according to standard procedures, and other sections were stained with 0.001% DAPI (4', 6-diamidino-2-phenylindole) in 0.01 mol/L phosphate buffered saline and kept away from light for 5 min at room temperature. These sections were observed and photographed by Olympus BX51 optical microscope.

## 2.3 Transmission electron microscopy (TEM)

Testes were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at 4℃. Specimens were then washed in the same buffer for three times, post-fixed in 1% $OsO_4$ in the same buffer for 2 h at 4℃. Fixed specimens were dehydrated in increasing ethanol concentrations and embedded into Epon812. Ultrathin sections about 70 nm were mounted on 200 or 300 mesh copper-rhodium grids and stained with lead citrate and uranyl acetate. Finally, the sections were observed and photographed by JEM-100SX electron microscopy.

# 3 RESULTS

## 3.1 Four phases of the nuclei of *G. gratiosa* during spermiogenesis

Each testis consists of a series of tubular follicles that are connected to the vas deferens. The spermiogenesis is underway in the follicular cyst where the spermatids undergo synchronous differentiation within the same cyst (Fig. 1: A – C; Fig. 2: A – C; Fig. 3: A – C; Fig. 4: A – C).

Numerous spermatids aligned parallel to one another are visible within the individual follicular cyst. During spermiogenesis, the nucleus undergoes dramatic changes that differentiate from small round into long and narrow. According to the morphology and structure of the nucleus, we divided the spermiogenesis into four phases: rounded phase (Fig. 1), leaf phase (Fig. 2), prismatic phase (Fig. 3) and mature phase (Fig. 4).

**3.1.1** The rounded phase of the nuclei during spermiogenesis: At the rounded phase, the nucleus is big and round, and the distribution of the genetic material within the nucleus is uneven (Fig. 1: A – C). The flagellum and the pro-acrosome appear at the late stage of this phase (Fig. 1: B). The ultrastructure of this phase shows that the nucleus is nearly rounded, the genetic material inside the nucleus is uneven and part of the chromatin is not thorough depolymerization (Fig. 1: D, E). Subsequently, the chromatin inside the nucleus progressively reorganized until the genetic material within the nucleus appears to a circular structure (Fig. 1: F) and finally changes into a uniform round (Fig. 1: G – I). A nearly rounded pro-acrosome and the Golgi complex appear in this phase. In addition the pro-acrosome is dense, and the cytoplasm is loose, but the nucleus is not dense (Fig. 1: F, H, I). The Golgi complex is located between the nucleus and the pro-acrosome, at the same time it constantly secretes the pro-acrosomal granule (Fig. 1: F, I).

**3.1.2** The leaf phase of the nuclei during spermiogenesis: At the leaf phase, the nucleus starts to elongate and condense, and its shape looks like a leaf (Fig. 2: A). The spermatids arrange in rows. The acrosomal complex looks like an inverted 'Y' at the tip of the nucleus and the flagellum is at the base of the nucleus (Fig. 2: B, C). The ultrastructure of this phase shows that the connection between spermatids clearly and the membrane structures of both sides of the nucleus have remarkable changes (Fig. 2: D). The pro-acrosome is close to the nucleus and the nucleus is dense (Fig. 2: E – G). Then the pro-acrosome extends to the both sides of the nucleus, and the front membrane of the cytoplasm develops to form an apical vesicle (Fig. 2: H). As differentiation proceeds, the acrosomal complex which consists of perforatorium and apical vesicle is clearly visible. The acrosome complex is

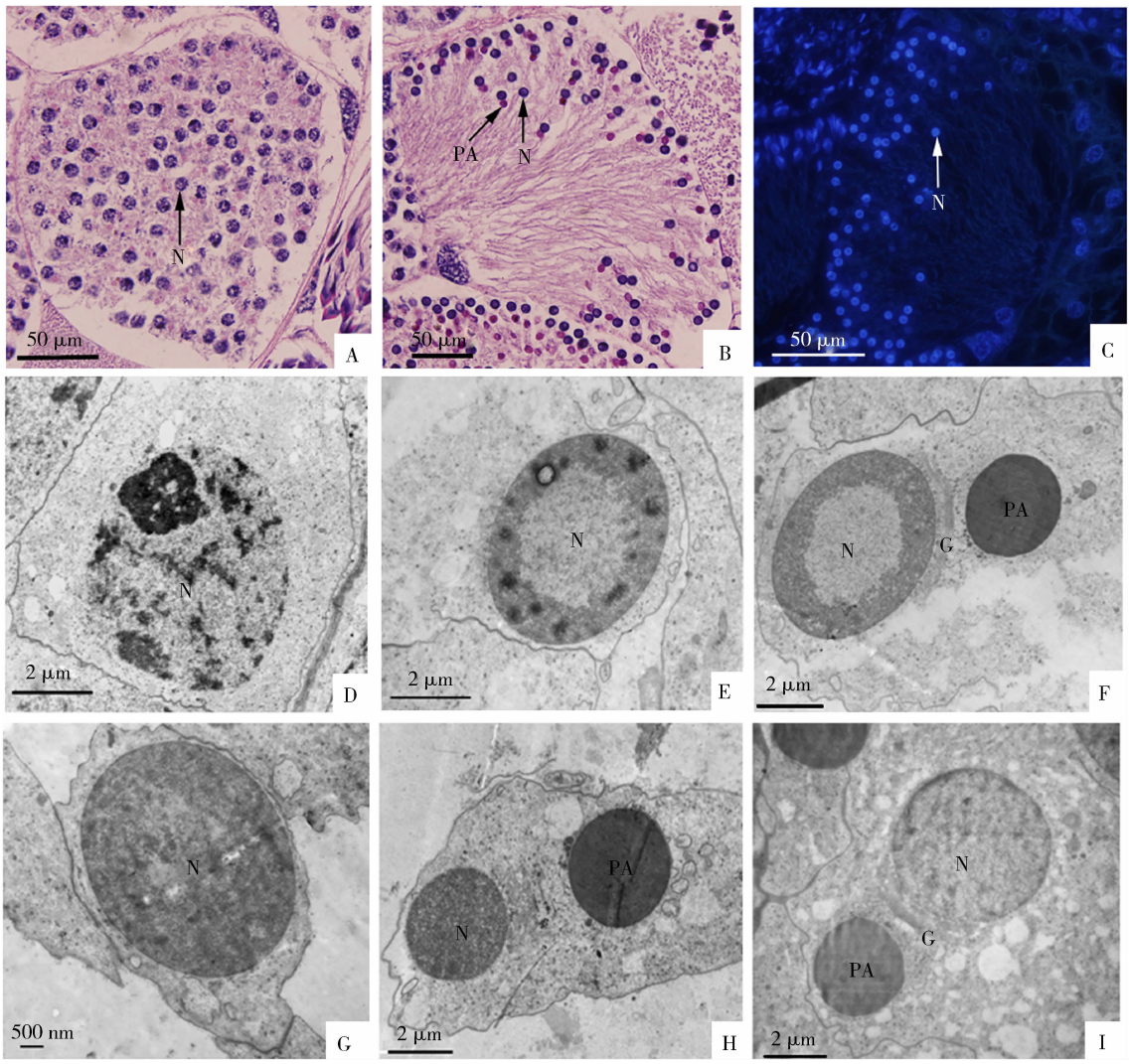


Fig. 1 The nucleus at the rounded phase during spermiogenesis of *Gampsocleis gratiosa*

A, B. Paraffin sections, H&E staining. A. Early stage, nuclei (N) are round and the distribution of the genetic material is uneven. B. Late stage, the flagellum and the pro-acrosome are formed. C. Paraffin section, nucleus (blue) marked with DAPI. D – I. Ultrastructure of nuclei. D. Early stage. E. Late stage. F. A rounded nucleus (N) and a nearly rounded pro-acrosome (PA). G. The nucleus is not dense. H. The pro-acrosome is dense and dyed darker. I. Golgi complex (G) is between the nucleus and the pro-acrosome, and constantly secrete the pro-acrosomal granule.

dense, located in the front of the nucleus and wrapping around the nucleus (Fig. 2: I). At the same time, the cross-section structure of the axoneme is visible (Fig. 2: D, F, H, I).

**3.1.3** The prismatic phase of the nuclei during spermiogenesis: At the prismatic phase, the nucleus continues to elongate and becomes narrower than before, and the shape is prismatic (Fig. 3: A, C). The spermatids become gathering (Fig. 3: B). The ultrastructure of this phase shows that the nucleus begins to elongate, and agglutination degree of the chromatin inside the nucleus is low, and electronic density is medium, while the acrosome complex is thick and dense. The membrane of the nucleus can be seen clearly (Fig. 3: D – I). Periphery of the nucleus is surrounded by many microtubules (Fig. 3: F). The cross-section structure of the axoneme is visible during this phase (Fig. 3: E, H, I).

**3.1.4** The mature phase of the nuclei during spermiogenesis: At the mature phase, the spermatids discard most of the material defined as the residual body to become mature spermatozoa, and the derelicts include the genetic material (Fig. 4: A, C). At the same time, the acrosome complex which is well developed looks like an arrow in the front of the nucleus, and a large number of spermatozoa hold together to form the spermatodesms (Fig. 4: B). The ultrastructure of this phase shows that the nucleus elongates and the genetic material within the nucleus undergo further agglutination to take on fibrous (Fig. 4: D, E, F, G). The genetic material within the end of part nucleus appears to be dispersedly distributed (Fig. 4: E). Electron density of the material in the nucleus is increased

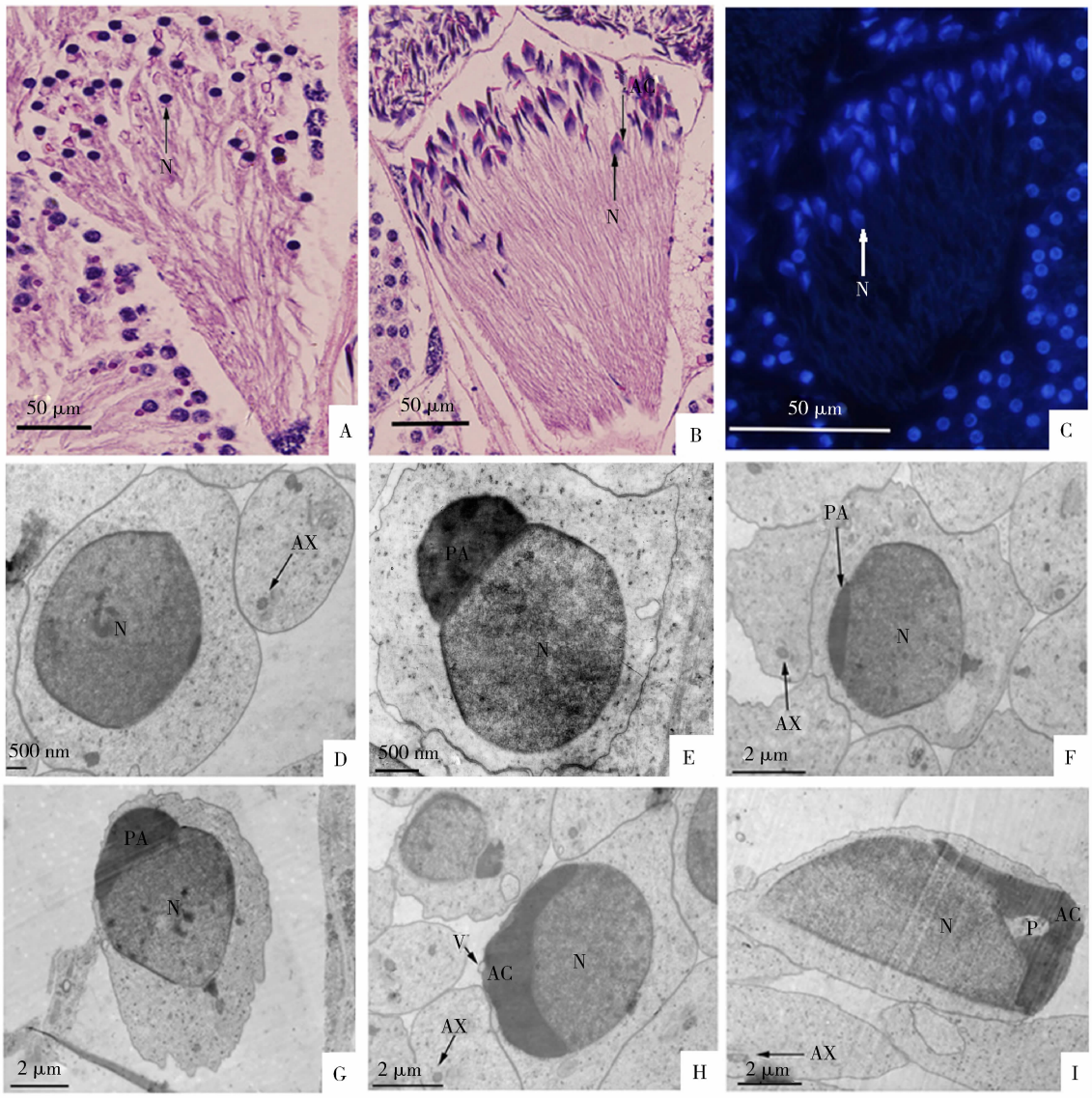


Fig. 2 The nucleus at the leaf phase during spermiogenesis of *Gampsocleis gratiosa*

A, B. Paraffin sections, H&E staining. A. Early stage, the shape of nucleus (N) looks like a leaf. B. Late stage, spermatids arrange in rows and the acrosome complex (AC) looks like an inverted Y. C. Paraffin section, nucleus (blue) DAPI marked. D – I. Ultrastructure of the nucleus. D. Membrane structures of the nucleus (N) have changes. E. Pro-acrosome (PA) move to the nucleus (N). F. Pro-acrosome (PA) close to the nucleus (N). G. Nucleus (N) is dense. H. Pro-acrosome (PA) expand on both sides of the nucleus. I. The acrosomal complex (AC) locates in the front of the nucleus and wrap around the nucleus. AX: Axoneme; P: Perforatorium; V: Apical vesicle.

and the longitudinal-section of elongated nucleus is pepper-shaped (Fig. 4: D, F). The acrosomal complex is around the nucleus and their periphery is surrounded by the dense material (Fig. 4: G), meanwhile the periphery of the acrosomal complex and nucleus are surrounded by microtubules (Fig. 4: E, G). Two oval-shaped mitochondrial derivatives locate behind the axoneme during this phase (Fig. 4: D, E). As the spermatid develops, spermatid turns into mature spermatozoa. The nucleus of spermatozoa which is surrounded by the acrosomal complex appears to pepper-shaped or cystic-shaped. The material within the nucleus has a uniform density, and the nucleus has a high electron density (Fig. 4: H). Then, many spermatozoa hold together to form the spermatodesms (Fig. 4: I).

### 3.2 Circular nuclei and round pro-acrosomes during the rounded phase of spermiogenesis of *G. gratiosa*

At the late stage of the rounded phase, two globular structures can be seen in the paraffin sections by H&E staining. One is a bluish-purple circular and the other is a red uniform round (Fig. 5: A). While only one blue structure can be seen in the paraffin section, marked by DAPI (Fig. 5: B).

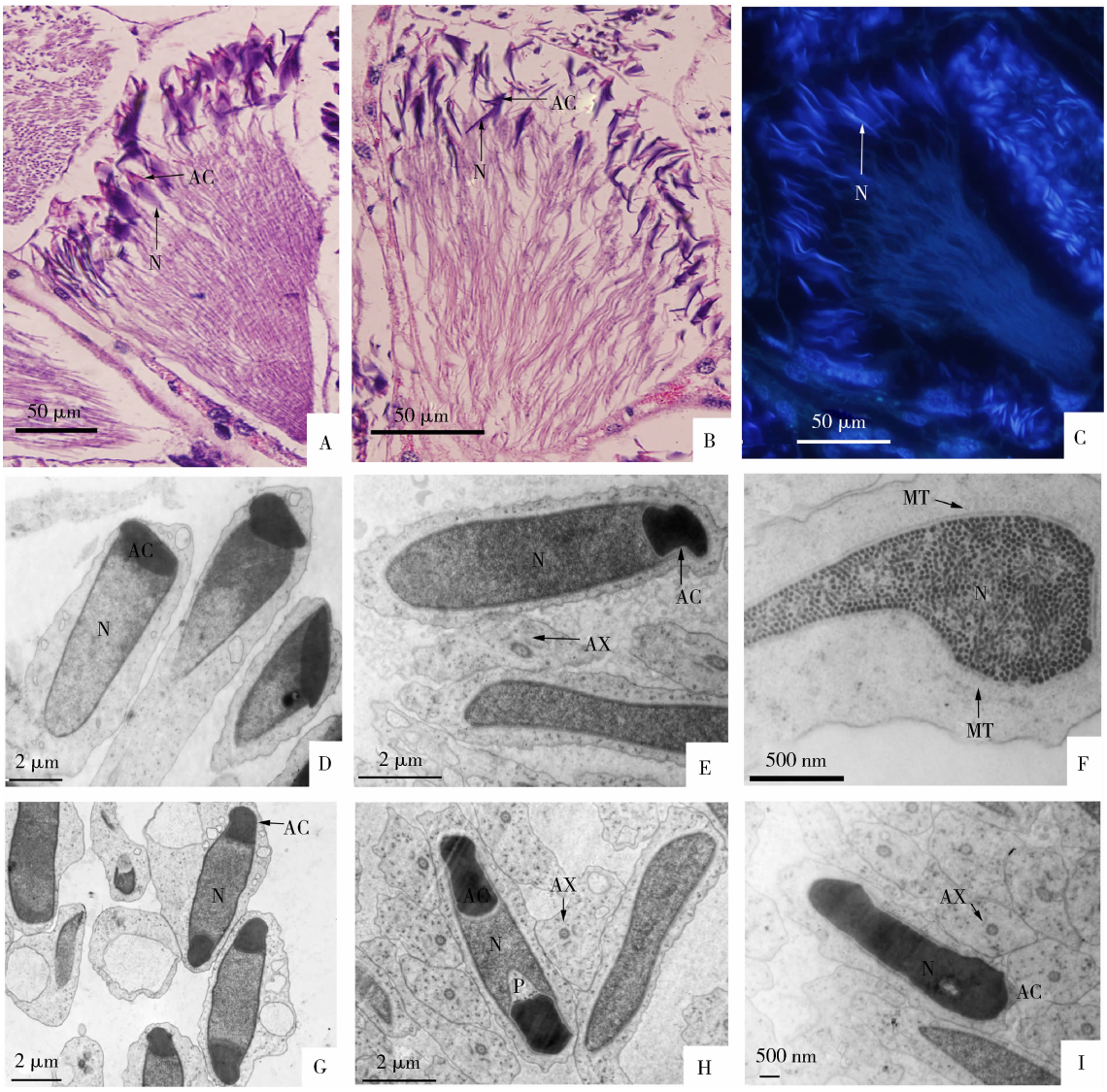


Fig. 3 The nucleus at the prismatic phase during spermiogenesis of *Gampsocleis gratiosa*

A, B. Paraffin sections, H&E staining. A. Early stage, the nucleus (N) is longer and narrower than before, its shape is prismatic. B. Late stage, spermatids gathered. C. Paraffin section, nucleus (blue) DAPI marked. D – I. Ultrastructure of nucleus. D. The chromatin inside the nucleus (N) is not agglutinated. E. Nucleus (N) began to elongate. F. Periphery of nucleus (N) is surrounded by microtubules (MT). G. The acrosomal complex (AC) is thick and dense. H. The acrosomal complex (AC) wrap around the nucleus (N). I. The cross-section structure of the axoneme (AX) is visible. AX: Axoneme; MT: Microtubule; P: Perforatorium.

So it can prove that in Fig. 5, the bluish-purple circular structures are nuclei and the red round structures are pro-acrosome. The ultrastructure shows the same result, and Golgi complex is emerged between the nucleus and the pro-acrosome (Fig. 5: C).

### 3.3 Residual bodies during the mature phase of spermiogenesis of *G. gratiosa*

At the mature phase, observations on the paraffin sections show that in the tail of spermatids many round structures form (triangular arrows in Fig. 5: D, E). These materials within the round structures are discarded by the spermatids which are defined as the residual body to become mature spermatozoa. As in the paraffin sections the nuclei are marked by DAPI, these structures are also seen (Fig. 5: E). So it can prove that the derelicts include the genetic material.

## 4 DISCUSSIONS

### 4.1 The nucleus dynamics during spermiogenesis of *G. gratiosa*

The spermiogenesis of *G. gratiosa* appears similar to that of other insects (Yamashiki and Kawamura, 1997; Fausto *et al.*, 2002; Sottile *et al.*, 2010; Viscuso *et al.*, 2012). Nucleus morphogenesis during spermiogenesis includes two major aspects: nucleus shaping and chromatin reorganization, and the nucleus shaping proceeds

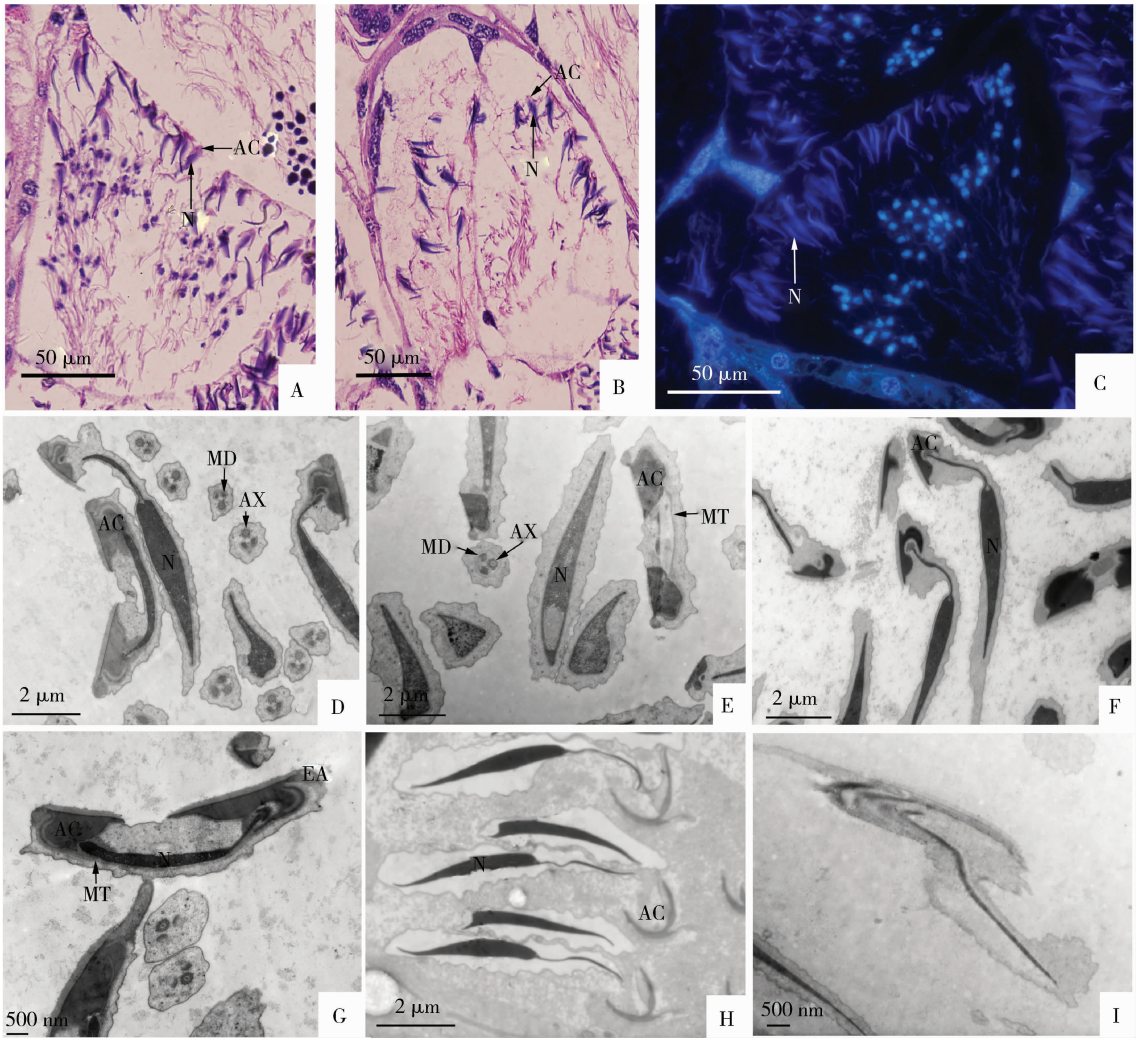


Fig. 4 The nucleus at the mature phase during spermiogenesis of *Gampsocleis gratiosa*

A, B. Paraffin sections, H&E staining. A. Spermatids discard most of the material. B. The acrosomal complex (AC) looks like an arrow, spermatozoa hold together to form the spermatodesms. C. Paraffin section, nucleus (blue) DAPI marked. D – I. Ultrastructure of nucleus. D. The genetic material undergoes further agglutination to take on fibrous. E. The genetic material appears to dispersible distribution. F. Longitudinal-section of the nucleus (N) is pepper-shaped. G. Periphery of the acrosomal complex (AC) and nucleus (N) are surrounded by microtubules (MT). H. The nucleus (N) has a high electron density. I. Spermatozoa hold together to form the spermatodesms. AX: Axoneme; EA: Exra-acrosomal layer; MT: Microtubule.

through several stages (Fabian and Brill, 2012). Based on the observations on the nucleus morphology and structure during spermiogenesis of *G. gratiosa*, it can be stated that this process includes rounded, leaf, prismatic and mature phases.

The role of microtubules in transformation of the sperm nucleus has been suggested in studies on many animal species (Yamashiki and Kawamura, 1997; Guerra and Esponda, 1999; Fabian and Brill, 2012). Nucleus shaping is driven by microtubules that emanate from the basal body and associate with the nucleus envelope. Nucleus associates with microtubules on the flattened surface, and these microtubules around nucleus organize into bundles, forming the dense body (Fabian and Brill, 2012). Several factors also have been implicated in regulating nucleus shaping through dense body cytoskeletal proteins (Li *et al.*, 1998; Vogt *et al.*, 2006). In the present study, behaviors of a nucleus and microtubules during spermiogenesis of *G. gratiosa* were observed by transmission electron microscopy (TEM) techniques. In the early rounded spermatids, the nuclear membrane has remarkable changes on both sides, and in the elongated spermatids the nucleus is surrounded by the microtubules (Fig. 3: F). It can be postulated that the nucleus shaping during spermiogenesis of *G. gratiosa* is driven by microtubules and other cytoskeletal proteins.

Duding spermiogenesis chromatin reorganization is concomitantly with nucleus shaping (Fabian and Brill, 2012). Chromatin reorganization involves switching from histones in the early rounded spermatid nucleus, first to transition proteins, then to protamines in the mature sperm nucleus (Rathke *et al.*, 2007; Johnson

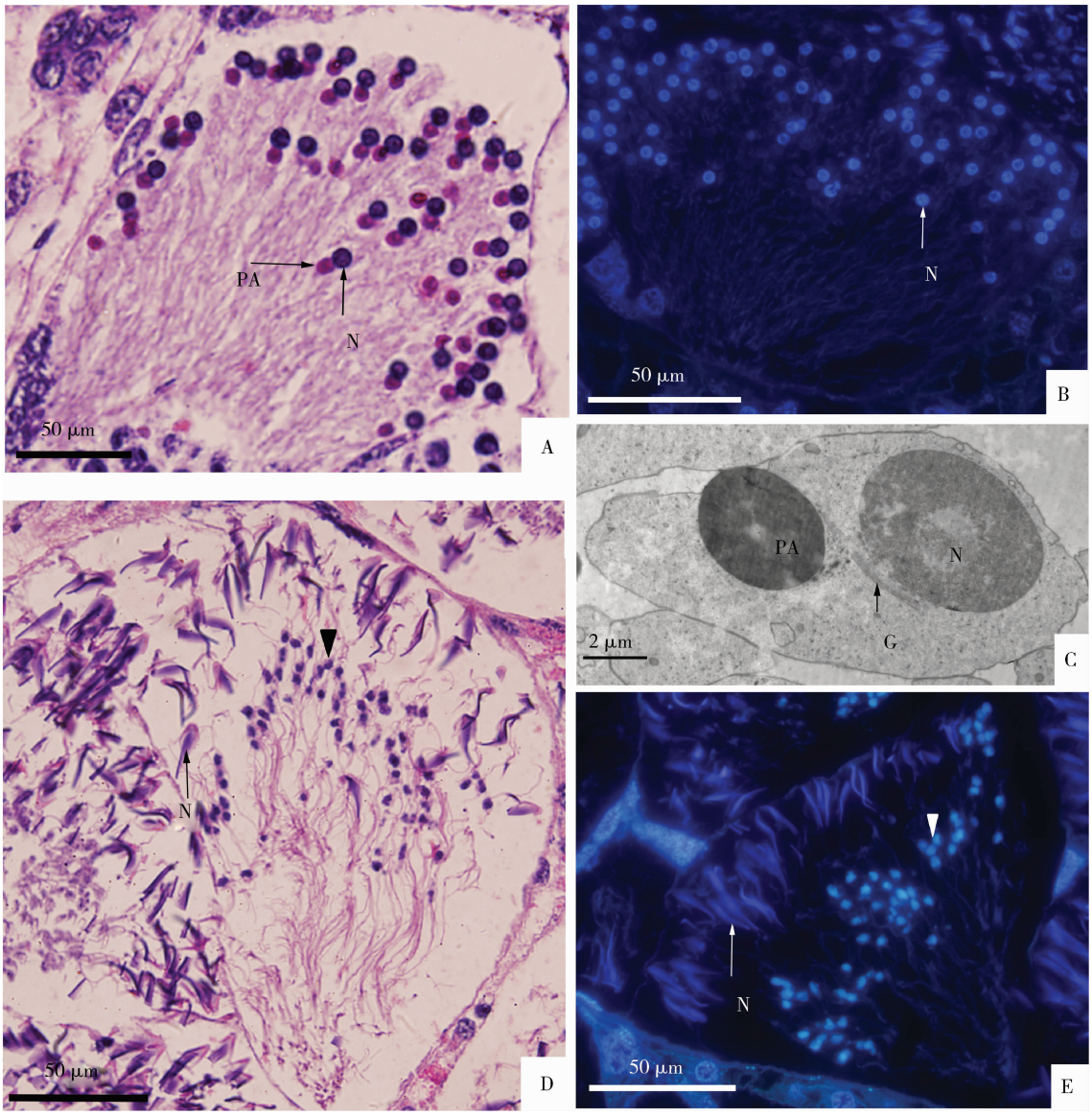


Fig. 5 The pro-acrosome at the rounded phase and the discarded material at the mature phase during spermiogenesis of *Gampsocleis gratiosa*

A, B, D, E. Paraffin sections, H&E staining. C. Ultrastructure of the circular nucleus (N) and the round pro-acrosome (PA). A. The bluish-purple circular structure is nucleus (N) and the red round structure is pro-acrosome (PA). B, E: Paraffin sections, nucleus (blue) DAPI marked. D, E: The residual body (triangular arrows) in the tail of mature spermatozoa. G: Golgi complex.

*et al.*, 2011; Kanippayoor *et al.*, 2013). In *Locusta migratoria* of Orthoptera, the differentiation process of spermiogenesis has been divided into ten developmental stages, and from stages 5 to 9 the chromatin rearrangment leads to a homogeneous compact and slender nucleus while the residual cytoplasm is sloughed off (Xi, 1995). During spermiogenesis of *G. gratiosa*, as a member of Orthoptera, the chromatin inside the nucleus also process continual reorganization, but it is poorly understand that whether protamines is instead of histones or not.

### 4.2 The pro-acrosomes and residual bodies during spermiogenesis of *G. gratiosa*

At the rounded phase during spermiogenesis of *G. gratiosa*, there are two remarkable globular structures in paraffin sections with H&E staining, but there is only one in the paraffin sections marked by DAPI (Fig. 5: A, B). H&E staining is short for hematoxylin-eosin staining, and hematoxylin stains the nuclei of cells blue to bluish-purple and eosin stains other cellular elements in the tissues from pink to red. DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent staining that binds strongly to A-T rich regions in DNA (Liu *et al.*, 2012). Acrosome formation had been studied in the house cricket, *Acheta domesticus*, also belonging to Orthoptera, by the use of vital dyes, histochemicaltests, and electron microscopy (Kaye, 1962; Kaye and

McMaster-Kaye, 1966). In the early spermatid of the house cricket there is a single cup-shaped Golgi body, called the acroblast. Then, a pro-acrosomal granule appears within the acroblast. The ultrastructure of the early spermatid in the katydid, *G. gratiosa*, shows that Golgi complex is located between the nucleus and the pro-acrosome (Fig. 5: A – C). Comparison of ultrastructure and microstructure, can prove that bluish-purple circular structures are nuclei and the red round structures are pro-acrosome.

During individualization of *Drosophila melanogaster*, the cystic bulges, called the cytoplasm droplets, move away from the sperm heads along the flagella and extrude syncytial cytoplasm and other morphogenetic debris in a 'waste bag' which is subsequently eliminated from the tail end of the cyst (Noguchi and Miller, 2003; Noguchi *et al.*, 2006, 2008). Similarly, there are some vesicle structures, the cytoplasm droplet, emerging in sperm flagella of *G. gratiosa* before forming mature sperm (Zhou *et al.*, 2012), and these vesicle structures are marked by DAPI (Fig. 5: D, E). Because DAPI is specific fluorescent probe, it could be speculated that the cytoplasm droplets include the genetic material that may be mtDNA. The cytoplasmic bulges including mtDNA move from the front to the tips of the tails along the flagella in Tettigoniidae during the late of spermiogenesis.

The information obtained so far only enables speculation on the dynamics of nucleus. Further research, possibly extending to other aspects of the reproductive biology of *G. gratiosa* involving the molecular mechanisms during spermiogenesis, is clearly needed.

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# 优雅蝈螽精子形成过程中核的形态结构变化观察

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**摘要:** 【目的】螽斯精子结构复杂,具有特征性的箭头状顶体,是研究昆虫精子形成的理想材料。为了研究螽斯精子形成过程中的动态变化机制,特别是细胞核的凝集机制和箭头状顶体的发生机制,本研究对优雅蝈螽 *Gampsocleis gratiosa* 精细胞和精子的细胞核进行了观察。【方法】选择发育良好的优雅蝈螽成虫精巢为研究材料,利用透射电镜技术、普通光学显微镜和荧光显微镜技术,制作光镜切片和电镜切片进行观察。【结果】根据其形态结构变化特征,将优雅蝈螽精子形成过程中的细胞核分为 4 个阶段:圆形核、叶形核、柱状核和成熟阶段。我们还通过常规 HE 染色,结合 DNA 特异性荧光探针 DAPI,证明了圆形核时期,精细胞内具有两个明显的球状结构,一个为细胞核,另一个是原顶体;精子成熟阶段,精子尾部排出的细胞质微滴中含有 DNA。【结论】优雅蝈螽精子形成过程中,精细胞的细胞核经历了显著的形态变化,精细胞核的形态变化与细胞骨架微管相关,细胞核塑形伴随着染色质的重组。本研究为进一步阐明直翅目昆虫精子形成的分子机制奠定了基础。

**关键词:** 直翅目; 优雅蝈螽; 细胞核; 原顶体; 超微结构; HE 染色; DAPI; 精子形成

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